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# Inhibition of Bacteriophage M13 Replication with Esterified Milk Proteins

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Esterified milk proteins [methylated (Met) or ethylated (Et) α-lactalbumin (ALA), β-lactoglobulin (BLG), and β-casein (BCN)], unmodified native milk proteins, and native basic proteins (calf thymus histone and hen egg white lysozyme) were tested for their antiviral activity against the bacteriophage M13 and for their influence on its replication (except BCN). All esterified milk proteins showed an antiviral activity against the bacteriophage M13, proportional to the extent of esterification and, hence, to the increased basicity of the modified proteins. Antiviral activity of 100% Met-BLG disappeared after its pepsinolysis but not after its trypsinolysis. The antiviral activity of Met-BLG was much higher than that of native basic proteins (histone and lysozyme). One hundred percent Met-BLG and 73% Et-BLG inhibited the replication of bacteriophage M13 completely, whereas 60% Met-ALA inhibited phage replication partially. Calf thymus histone inhibited the replication of bacteriophage M13 at a lower extent (20%) than Met- and Et-BLG (100% inhibition). Protein concentration, pH, and concentration of the *Escherichia coli* culture in the preincubation medium of the virus were other factors influencing antiviral activity. Interactions of esterified proteins with the phage DNA (phenol extracted) followed the same pattern as observed during studies of the inhibition of the phage replication: Met-BLG > Et-BLG ≥ Met-ALA.

**KEYWORDS: Milk proteins; esterification; phage; antiviral activity**

## INTRODUCTION

DNA-binding proteins can have antiviral and tumor suppressor activities (*1*, *2*). Tumor suppressor protein p53 was found to bind preferentially to supercoiled DNA (*3*). Specific cellular endogenous proteins were observed to inhibit viral replication: human MxA protein inhibits the replication of influenza C virus (*4*), and α 1-PDX protein was found to interfere with viral replication of HIV-1 (*5*). Milk proteins were already reported to have antiviral effects, which were attributed particularly to lactoferrin (*6*, *7*), a mammalian iron-binding protein of 80 kDa (*8*). Human lactoferrins exhibit potent antiviral activities against cytomegalovirus (*6*, *7*) and HSV-1 infection (*9*, *10*) and inhibit the replication of HIV-1 in a T-cell line if they are added prior to infection or during the virus adsorption step (*11*).

Antiviral and antitumor activities are correlated to each other. Factors that enhance protein−DNA cross-linking, for example, the antibiotic Givocarcin V, show antiviral and antitumor activities (*12*, *13*). RNA− and DNA−protein interactions are essential for some pathogenic viruses. Tat protein is essential for HIV viral replication and activates transcription by binding to the transactivation-responsive (TAR) site located at the 5′-end of the viral transcript. In hepatitis C virus (HCV), a ∼350 nucleotide region in the 5′-end of the viral transcript is required for cap-independent translation and is vital for virus replication (*14*). Hence, the disruption of such interaction with external nucleic acid binding proteins can result in significant reduction of virus replication and infection.

The potential capacity of some basic proteins [hen egg white lysozyme and three of its basic derivatives (guanidyl, methyl ester, and guanidyl methyl ester)] to interact with herpes simplex type 1 replication was previously studied (*15*). Whereas, according to Cisani et al. (*15*), the native enzyme displayed only modest anticytopathic activity, the basic derivatives exhibited a markedly enhanced activity. It should be highlighted, however, that, albeit raising basicity, in this case the modifications of lysozyme are of different chemical character. The increase of basicity was reflected by a marked increase of antiherpes activity. The formation of syncithia in cell monolayers infected with a macroplaque strain of herpes simplex virus was found to be inhibited by hen egg white lysozyme. Inhibition was roughly proportional to the lysozyme concentration. The virus titers in supernatant fluids of lysozyme-treated cultures were

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also reduced compared with untreated cultures (*16*). Lysozyme antiviral activity was dependent on its positive charge and three-dimensional structure because the inactivated enzyme retained its antiviral activity (*15*).

DNA-binding properties of esterified milk proteins were studied previously, showing that interactions resulted from the increase of the net positive charge basifying the modified proteins (*17*). Hence, it was of interest to verify the possible use of esterified milk proteins as inhibitors of viral replication. Because esterified milk proteins bind to DNA in a nonspecific way (*17*), they are endowed with broad specificity against viruses, whatever their viral mutated forms could be. This could be of interest because viruses mutate frequently to escape immune control (*18*), producing viral proteins no longer recognizable by host antibodies (*19*, *20*). Bacteriophage M13, which is specific for *Escherichia coli*, is a well-characterized filamentous 6407-nucleotide single-stranded bacteriophage (*21*). Its structure and its mechanism of replication are well-known (*22*−*24*). Hence, it can be used as an excellent model phage for study of the antiviral action of esterified milk proteins.

## MATERIALS AND METHODS

$\beta$-Lactoglobulin (BLG) was purchased from Armor Proteins (Saint-Brice-en-Coglès, France) and purified according to the method of Mailliart and Ribadeau Dumas (*25*). $\alpha$-Lactalbumin (ALA) (Armor Proteins) was further purified by ion-exchange chromatography using a DEAE-Sepharose Fast Flow column (50 × 300 mm). The elution was performed with a 50 mM Tris−10 mM $CaCl_2$ buffer at pH 8, by using two steps at 10 and 40 mM NaCl. $\beta$-Casein (BCN) (purity > 95%) was purchased from Lactalis (Retiers, France) and used without further purification. Hen egg white lysozyme (EC 3.2.1.17), calf thymus histone type II-AS, bovine trypsin (EC 3.4.21.4 with activity of 10000 BAEE/mg), porcine pepsin (EC 3.4.23.1, 4550 units/mg), and M13 mp 18 bacteriophage were purchased from Sigma. *E. coli* TG1 was obtained from BioLabs and kept on M9 minimal medium (*22*). Plate count agar (PCA) was from Biokar Diagnostics, Beauvais, France. All solvents and chemical reagents were of analytical grade.

**Protein Esterification.** The procedure of Sitohy et al. (*26*) was used as follows. BLG, ALA, or BCN was esterified by dispersing an amount in concentrated (>99.5%) methanol or ethanol. Amounts of hydrochloric acid equivalent to 50 molar ratio (MR, mole acid/mole carboxyl group) were added dropwise at the start of the reaction time. All of the reaction mixtures were kept at 4 °C under continuous stirring. At the end of the reaction (6 h), the samples were centrifuged at 10000*g* for 10 min. The resulting supernatant was discarded, and the residue was dispersed in a volume of alcohol (99.7% ethanol) equal to that of the discarded supernatant and well mixed before recentrifugation in the same conditions. This washing step was repeated three times. The final precipitate was dissolved in an appropriate amount of distilled water and kept at −80 °C until freeze-drying. The lyophilized samples were kept at −20 °C until analysis. To quantify the extent of esterification of proteins, the color reaction using hydroxylamine hydrochloride developed by Halpin and Richardson (*27*) was used with a modification according to Bertrand-Harb et al. (*28*).

**Peptic and Tryptic Hydrolysis of Methylated (Met-)BLG.** Weighed amounts (10 mg/mL) of native and Met-BLG were dispersed in 20 mM citric acid buffer, pH 2.6. Appropriate aliquots of pepsin solution (initial concentration = 2 mg/mL of $H_2O$) were added to give an enzyme/substrate (E/S) ratio of 2%. The reaction mixture was incubated at 37 °C for 5 h. An equal volume of 0.2 M phosphate buffer, pH 7, was added to stop the proteolysis. In the case of tryptic hydrolysis, similar conditions were used except that the reaction buffer was 50 mM Tris at pH 7.1 and the reaction time was 20 h. At this time, maximum trypsinolysis is obtained (*29*). The reaction product was kept at −20 °C until testing.

**Degree of Hydrolysis (%DH).** The %DH was determined by quantifying the increase in free amino groups in the resulting hydrolysates by the reaction with *o*-phthalaldehyde (OPA) by measuring the absorbency at 340 nm (*30*). The %DH was computed by dividing the amount of liberated amino groups by the total amount of bound $\alpha$-amino groups in the starting substrate and multiplying by 100.

**Influence of Esterified Proteins on Viral Activity.** The following procedure was adopted with some modifications, which will be indicated under Results. Aliquots (10 $\mu$L) of protein solutions (original concentration = 360 $\mu$M) were mixed with equal volumes of virus stock [$10^6$ plaque-forming units (pfu)/mL] and an activated bacterial culture (2 h old). The mixtures were incubated at 37 °C for 30 min to obtain the maximal action of protein before dilution with the addition of 250 $\mu$L of *E. coli* culture (2.5 h old) and 3 mL of Luria Broth (LB) medium containing 0.7% agar (kept at 50 °C) and then pouring the resulting mixture on the top of the PCA medium in Petri dishes warmed to 37 °C. A positive control was similarly formulated but in the absence of the tested proteins. After one night of incubation, Petri dishes were observed and scanned for the viral inhibition zones. The relative reduction in the number of these inhibition zones relative to the positive control was taken as a measure of the antiviral effect of the tested proteins. All plaque assays have been done at least in duplicates and showed reproducible results.

**Influence of Esterified Proteins on Virus Replication.** Aliquots (10 $\mu$L) of protein solutions (360 $\mu$M) were mixed with equal volumes of virus stock ($10^6$ pfu/mL) and of activated bacterial culture (2 h old) and incubated for 30 min at 37 °C (first infection medium). Then, a further 250 $\mu$L of *E. coli* culture (2.5 h old) was added to the incubated mixture and kept at 37 °C for another 30 min (second infection medium). Additional fresh LB medium (0.75, 1, and 8 mL) was added to the incubated mixture after 1, 2, and 3 h (from the end of the second incubation time), respectively. The last mixture was incubated at 37 °C so that the total incubation time was 18 h. Positive control was performed similarly but in the absence of tested protein, and negative control was obtained in the absence of both tested proteins and virus. The final mixtures were centrifuged at 12000 rpm to remove the bacterial cells. Two milliliters of 20% PEG 8000 containing 2.5 M NaCl was added to the supernatant. After 15 min, the mixtures were centrifuged for 10 min at 18000*g*. The obtained precipitate was dissolved in 1 mL of 1× TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA) and combined with 0.5 mL of phenol saturated with 10 mM Tris-HCl, pH 8, and 1 mM EDTA. After centrifugation at 18000*g* for 5 min, the aqueous phase (0.9 mL) was recovered, and viral DNA was precipitated by the addition of 3 mL of a mixture of 99.7% ethanol and 3 M sodium acetate (25:1, v/v), previously kept at −20 °C. After centrifugation at 18000*g* for 10 min, the resulting precipitate was washed with 70% ethanol kept at −20 °C. After recentrifugation in the same conditions, the final precipitate was dried under vacuum before being dissolved in 1 mL of 1× TBE buffer (89 mM Tris−borate, 2 mM EDTA, pH 8). The optical density was then measured at 260 nm. The amount of nucleic acid was calculated as $OD_{260} = 1$, equivalent to 50 $\mu$g/mL. The final solution was diluted four times with 1× TBE buffer, and 10 $\mu$L of the diluted solution was used for electrophoresis on 0.6% agarose.

**Interactions between Viral DNA and Esterified Proteins.** Produced viral DNA was extracted as described above except that no protein was added. The concentration of the obtained DNA was calculated by measuring the optical density at 260 nm, and then the sample was diluted to a concentration of 200 ng/$\mu$L. A 5 $\mu$L sample of such a solution was added to different volumes of esterified proteins (42 $\mu$M) to give basic amino acids/phosphate groups in DNA ratios of 0:1, 0.5:1, 1:1, 2:1 and 3:1, and then the reaction mixtures were completed to a final volume of 20 $\mu$L with 1× TBE buffer. Reaction mixtures were kept at room temperature for 20 min before analysis by electrophoresis on 0.6% agarose.

## RESULTS AND DISCUSSION

**Inhibitory Effect of Esterified Proteins on Viral Activity.** To investigate the inhibitory effect of esterified proteins on viral activity, a simple technique was developed in this study as described under Material and Methods (Influence of Esterified Proteins on Viral Activity). During the 30 min preincubation period at 37 °C, the virus is supposed to expose some regions

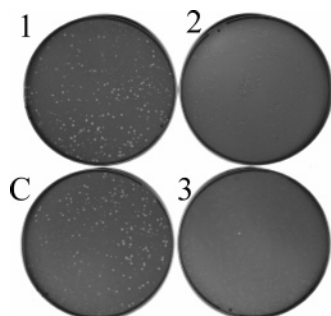


**Figure 1.** Viral activity of bacteriophage M13 in the presence of BLG: (1) native, (2) 100% Met-BLG, and (3) 73% Et-BLG. (C) is control in the absence of protein. Equal volumes (10 $\mu$L) of virus stock solution ($10^6$ pfu/mL), *E. coli* (2.5 h culture), and proteins (360 $\mu$M) were incubated at 37 °C for 30 min before the addition of 250 $\mu$L of *E. coli* (3 h culture) and 3 mL of LB medium containing 0.7% agarose. Spreading was made on the top of PCA medium.

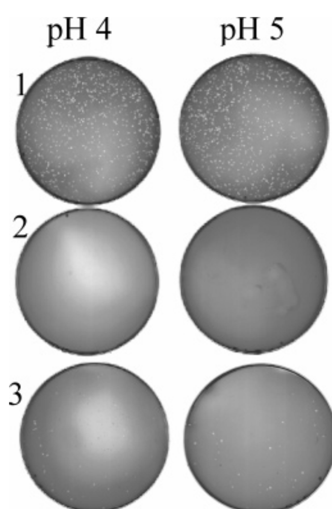


**Figure 2.** Viral activity of bacteriophage M13 in the presence of ALA: (1) native, (2) 60% Met-ALA, and (3) 36% Et-ALA. Protein samples (360 $\mu$M) were dissolved at pH 4 or 5. Virus stock content was $10^8$ pfu/mL.

of its genomic DNA to the interacting external tested proteins (*31*). The volume of *E. coli* is kept minimal to avoid dilution and an eventual hydrolytic action of *E. coli* proteases on esterified proteins. The inhibitory activity of esterified BLG and ALA was observed through the diminution of the inhibition zones induced by the virus on the host *E. coli* culture spread over Petri dishes. All esterified protein samples showed antiviral inhibitory effects, which were higher in the case of 73−100% esterified BLG (**Figure 1** [3 and 2, respectively]) than in the case of 36−60% esterified ALA (**Figure 2** [3 and 2, respectively]), which demonstrates that the esterification rate is the main factor involved in the inhibitory effect. In another experiment, when tested esterified proteins were added to virus stock solution at a 1:1 volume ratio (10 $\mu$L of each) in the absence of *E. coli* culture during the preincubation period, a similar antiviral inhibitory effect of esterified proteins was observed. Such conditions do not reproduce the real situation because the active virus is always associated with the host bacteria. However, this result demonstrates that the action of esterified proteins is directly oriented against the virus and does not act through an indirect influence on the host bacterial cells because no bactericidal activity was observed in control experiments (data not shown). Otherwise, incubating equal volumes of protein solution (360 $\mu$M) and *E. coli* culture (10 $\mu$L of each) for the same preincubation period before a further addition of 250 $\mu$L of *E. coli* culture, then completing up to 2 mL with fresh LB medium and extending the incubation at 37 °C overnight, did not lead to any inhibitory effect of esterified proteins on the growth of bacteria. Consequently, it might be concluded that the action of esterified proteins is directed against the virus only.

Native BLG showed no effect on viral activity. The main inhibitory effect was obtained in the case of 100% Met-BLG and 73% ethylated (Et-)BLG, which totally suppressed the inhibitory action of the bacteriophage M13 on the growth of *E. coli* when proteins were used at a concentration of 120 $\mu$M in the preincubation medium (**Figure 1**). A relatively lower antiviral effect was observed in the case of 60% Met- and 36% Et-ALA. Met- and Et-ALA derivatives inhibited about 93 and 33%, respectively, of the viral activity when the tested protein samples were dissolved at pH 7. When the pH of the tested proteins was adjusted to pH 7, the esterified ALA samples were only 60% soluble (*32*) because their isoelectric points (p*I*) are close to this value (p*I* of 9 and 8 in the case of 60% Met- and 36% Et-ALA, respectively) (*26*). Consequently, because their concentrations decreased in solution, their antiviral activities were decreased. Additionally, the basicity of esterified ALA is lower than that of esterified BLG derivatives, which have p*I* of 10 and 9.5 in the case of 100% methylated and 73% ethylated derivatives, respectively (*26*). Protein basicity was found to be the main factor during the interaction of esterified proteins with nucleic acids and during the inhibition of in vitro DNA replication (*33*, *34*). Consequently, this factor may also be responsible for the interaction of esterified proteins with viral DNA and, hence, inhibiting its activity. Esterified BCN samples (100% Met- and 56% Et-) were totally devoid of antiviral activity at pH 7 because they were less soluble and less basic than 60% Met-ALA (p*I* 7−8) and 36% Et-ALA (p*I* 5.5−7.0). Their low solubility at this pH could be due to the fact that they are more hydrophobic than their native forms.

**Factors Influencing Antiviral Activity.** *Effect of pH.* Because of the relatively low antiviral inhibitory activity of esterified ALA and esterified BCN at pH 7, their activity was further measured at lower pH values, at which they are nearly 100% soluble (*32*). Data in **Figure 2** show that 60% Met-ALA dissolved either at pH 4 or at pH 5 inhibited the replication of the bacteriophage M13 totally, whereas 36% Et-ALA did it partially. For the sake of comparison, native and esterified BLG samples were also tested for their antiviral activities at pH 4−5, showing a total inhibition of the viral activity in this pH range in the case of esterified samples, whereas native BLG did not show any inhibitory action (data not shown). Because both esterified samples of two different milk proteins studied are soluble in this range of pH, the observed differences in their antiviral activity should be only due to differences in protein basicity due to their different extents of esterification. For example, 73% Et-BLG was characterized by a p*I* of 9.5 as compared to a p*I* of 8 in the case of 35% Et-ALA.

Modified BCN samples showed no antiviral effect at pH 7, at which they are nearly insoluble. Lowering the pH to 5 did not improve the antiviral activity, and further lowering of the pH to 4 endowed 100% Met-BCN with an antiviral activity (98% of inhibition of the viral activity (**Figure 3**). However, 56% Et-BCN derivative was still inactive at pH 4 because of its low basicity (p*I* of 5.5−7.0 still below that of Et-ALA).

*Effect of Protein and E. coli Culture Concentrations.* When Met-BLG was present in the preincubation medium at a concentration of 120 $\mu$M, a total inhibition of viral activity was observed. Diluting the protein concentration to 60 $\mu$M reduced

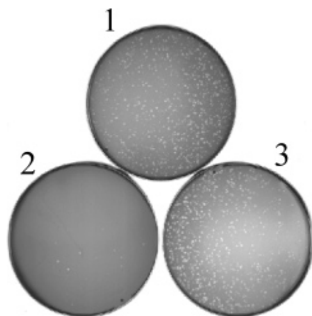


**Figure 3.** Viral activity of bacteriophage M13 in the presence of BCN: (1) native, (2) 100% Met-BCN, and (3) 56% Et-BCN. Protein samples (360 $\mu$M) were dissolved at pH 4. Virus stock content was $10^8$ pfu/mL.

the antiviral activity to ≈75% of the original level. Further dilutions of protein concentration decreased gradually the antiviral activity of the protein. Consequently, to obtain the highest antiviral activity, esterified protein concentration in the preincubation medium should not be lower than 120 $\mu$M.

Increasing the ratio of *E. coli* culture in the preincubation medium decreased gradually the antiviral activity of Met-BLG. The most favorable ratio of *E. coli* culture was 33.3% of the preincubation medium, conditions under which a total viral inhibition was obtained in the presence of 120 $\mu$M protein. Increasing the ratio of *E. coli* culture to 55% of the preincubation medium consequently resulted in a decrease of the protein concentration in the medium to 84 $\mu$M and in a decrease of the antiviral activity to 70% of its original level. A further increase of the ratio of *E. coli* culture to 71% of the preincubation medium diluted protein concentration to 51 $\mu$M and lowered the antiviral activity to 33% of its original value. Finally, increasing again the ratio of the added *E. coli* culture up to 88% of the preincubation medium lowered the protein concentration to 21 $\mu$M and resulted in a complete disappearance of the antiviral activity of the modified protein (data not shown). The rapid decrease of the antiviral activity of esterified protein by increasing the ratio of the *E. coli* culture in the preincubation medium is mainly due to the parallel dilution of the tested protein and possibly due to the proteolysis of esterified proteins by the bacteria.

*Effect of Peptic and Tryptic Hydrolysis.* Peptic hydrolysis of Met-BLG for 5 h reduced its antiviral activity to ≈40% of its original level. Tryptic hydrolysis of the same substrate during 20 h did not suppress its antiviral activity (data not shown). The loss of antiviral activity after pepsinolysis of Met-BLG might be due to the loss of DNA-binding activity of the protein as previously observed (*35*), which results from its extensive hydrolysis (25% DH), leading to very small peptic fragments (<1 kDa) (*36*) unable to form stable complexes with DNA. In contrast, trypsinolysis for 20 h of Met-BLG resulted in a low degree of hydrolysis (7% DH) and in peptide populations of molecular masses spread in the range of 3−18 kDa (*37*), which were still able to interact with DNA, showing in parallel powerful antiviral activity. This relationship between DNA-binding capacity of esterified proteins and their antiviral activity may give an insight on the mechanism by which these modified proteins are exerting their antiviral activity.

**Comparison of the Antiviral Activity of Met-BLG with That of Native Basic Proteins.** The antiviral activity of 100% Met-BLG was estimated to be total when protein was added in the preincubation medium at a concentration of 120 $\mu$M to an initial virus amount of $10^6$ pfu/mL. The same inhibitory activity was observed when using more highly concentrated ($10^8$ pfu/mL) virus stock solution. In these conditions, native basic

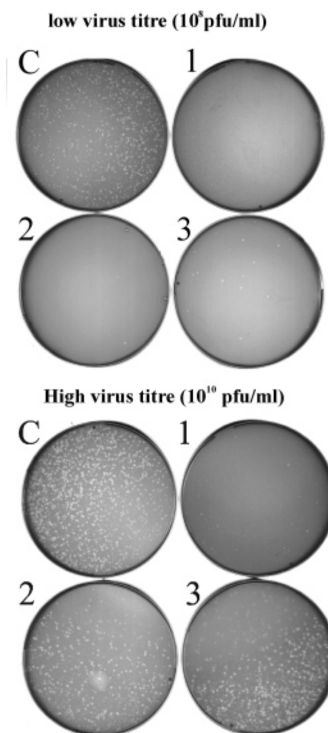


**Figure 4.** Comparison between the antiviral activity of (1) 100% Met-BLG, (2) calf thymus histone, and (3) chick egg white lysozyme against bacteriophage M13 in low ($10^8$) or high ($10^{10}$ pfu/mL) content. (C) is the control in the presence of native BLG.

proteins (hen egg white lysozyme and calf thymus histone) showed a similar antiviral effect (97−99% of inhibition) as compared with Met-BLG. When the experiment was performed with the same protein concentration but using higher virus stock concentration ($10^{10}$ pfu/mL), important differences were observed between Met-BLG and native basic proteins (**Figure 4**). Antiviral activity of Met-BLG was maintained near 100%, whereas lysozyme and histone lost most of their antiviral activity, showing maximum inhibitory activities of 50 and 25%, respectively. These relatively lower antiviral activities of native basic proteins might be due to the existence in the virus of a mechanism recognizing native basic cellular proteins and avoiding their inhibitory activity. Another explanation should be that the complexes formed between Met-BLG and the DNA of the virus are more stable toward cellular digestion, as was previously observed during in vitro pepsin and DNase digestion (*33*), despite the demonstrated susceptibility of esterified proteins to pepsinolysis (*35*). Esterified proteins being highly resistant to tryptic hydrolysis (*37*), their complexes with DNA should be rather resistant to the eventual attack by bacterial trypsin-like enzymes. The hydrophobic nature of the complexes between Met-BLG and DNA of the virus due to the presence of the grafted methyl groups might increase their stability against cellular digestion. The increased hydrophobicity may also increase the stability of the DNA-esterified proteins complexes, consolidating their antiviral activity. Consequently, it could be supposed that the antiviral activity of esterified proteins is not only due to their increased basicity, as native (unmodified) proteins are less efficient, but also due to their increased hydrophobicity, which might render their complexes with DNA of the virus more resistant to hydrolytic action. In previous works, Cisani et al. (*15*, *16*) observed a modest antiviral activity of native hen egg white lysozyme against herpes simplex virus,

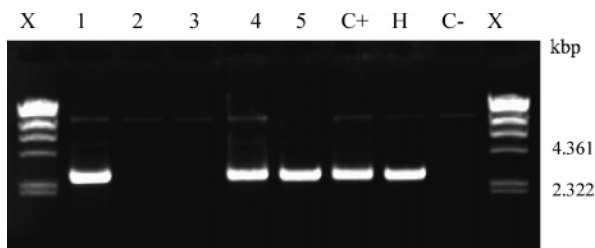


**Figure 5.** Agarose gel electrophoresis of synthesized DNA of bacteriophage M13. Bacteriophage was in the presence of (1) native BLG, (2) 100% Met-BLG, (3) 73% Et-BLG, (4) native ALA, (5) 60% Met-ALA, and (H) calf thymus histone. (C+) is positive control (virus in the absence of proteins) and (C–) is negative control (in the absence of virus). (X) is a size marker.

accentuated by increasing positive charge of the enzyme through chemical modification (guanidination, methylation).

**Effect of Inhibitory Activity of Esterified Proteins on Bacteriophage Replication.** Bacteriophage replication was followed by measuring the quantity of viral DNA formed after 18 h of incubation with *E. coli* in the presence of esterified proteins. Incubation of 10 $\mu$L of virus stock solution ($10^6$ pfu/mL) with 250 $\mu$L of a 2 h *E. coli* culture in a final volume of 10 mL of LB medium produced a total amount of 17 $\pm$ 0.6 $\mu$g of DNA. Addition of 120 $\mu$M native BLG or native ALA to the preincubation medium slightly enhanced the amount of synthesized DNA up to 20 $\pm$ 1 $\mu$g. In contrast, nearly no DNA was formed in the presence of Met- or Et-BLG. It can be concluded that the presence of Met- and Et-BLG inhibited totally the viral replication. In the presence of Met-ALA, 13 $\pm$ 0.5 $\mu$g of DNA was produced only, as compared with 20 $\pm$ 1 $\mu$g formed in the presence of native ALA. In the same conditions, 20% of inhibition of replication only was observed, as compared with the value obtained in the presence of native BLG or native ALA. This result agrees with the previous observation that the antiviral activity of native protein was much smaller than that of Met-BLG.

The electrophoretic patterns of the obtained viral DNA preparations (**Figure 5**) confirm the previous conclusions. In the presence of 100% Met-BLG or 75% Et-BLG, the band representing the synthesized bacteriophage DNA is completely absent, which demonstrates a complete inhibition of viral replication. In the presence of 60% Met-ALA or native histone, the intensity of the band representing the viral DNA was still important, albeit lower than that obtained in the presence of native ALA, demonstrating a slight inhibitory effect of these proteins on the viral replication.

In conclusion, inhibition of viral replication by esterified milk proteins is highly associated with the antiviral activity of these proteins. Viral replication is probably stopped by interactions of basic esterified proteins with exposed domains of the viral DNA, inhibiting its subsequent replication. Initiation of cellular or viral DNA replication is activated by the binding of origin-binding proteins (OBPs) to specific sequences within the DNA (*38*, *39*). The competition between OBPs and esterified proteins for such sequences may suppress viral replication. When an M13 rod-shaped virus penetrates the bacterial pilus, its coat protein (the product of gene III) is removed and the infecting single-stranded DNA is converted by cellular enzymes into a double-stranded circular form, which represents the starting point of virus replication (*40*). Esterified milk proteins could possibly bind to the single-stranded DNA, preventing the formation of the double-stranded form and, consequently, blocking the subsequent replicating events. Esterified milk proteins were previously proved to inhibit in vitro DNA replication (*34*) as a result of the DNA-binding properties of such proteins.

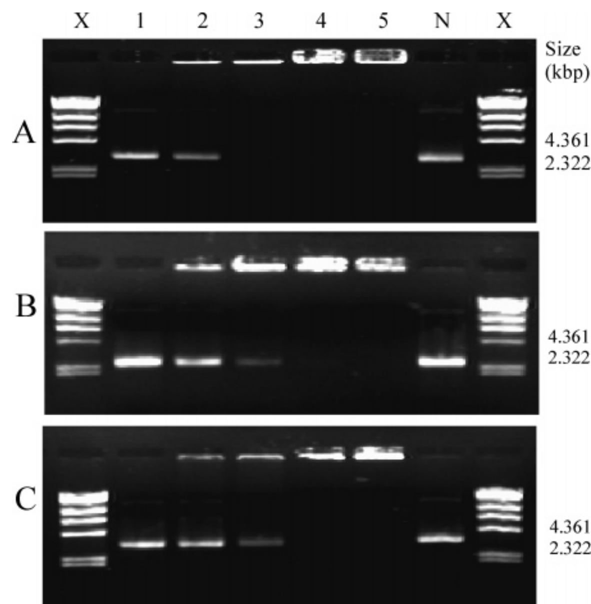


**Figure 6.** Interactions in vitro between DNA extracted from bacteriophage M13 and (**A**) 100% Met-BLG, (**B**) 73% Et-BLG, and (**C**) 60% Met-ALA. Different ratios of protein positive charges/DNA negative charges were used, as follows: (1) 0:1, (2) 0.5:1, (3) 1:1, (4) 2:1, and (5) 3:1. (X) is a size marker; (N) is positive control (virus in the absence of proteins).

The present results confirm our previous observations suggesting a mechanism of inhibition similar to that observed in case of in vitro DNA replication (*34*). The relatively low inhibitory effect of histone on viral replication despite its high basicity may be due to the fact that viral DNA, together with viral proteins and cellular histones, are assembled into virions in the host cells nuclei (*41*−*43*); that is, the virus is able to escape histone perturbation of its replication and propagation.

**Interactions of Viral DNA with Esterified Proteins.** Phenol-extracted bacteriophage DNA was allowed to interact with Met- and Et-BLG, Met-ALA, and the native forms of these whey proteins, placed at different ratios of protein positive charge/DNA negative charge. To prepare the starting proteins in a completely soluble state, the pH was adjusted to 7.0, 6.5, and 5.0 in the case of Met-BLG, Et-BLG, and Met-ALA, respectively. The obtained complexes were analyzed by agarose gel electrophoresis (**Figure 6**). The three esterified proteins could form a complex with the viral DNA, preventing its migration in the gel, whereas the native forms of these proteins had no effect. However, the capacity of esterified proteins to make a complex with DNA was relatively higher in the case of 100% Met-BLG than in the case of 73% Et-BLG or 60% Met-ALA, demonstrating the main role played by the esterification rate in the inhibitory activity of esterified derivatives. In the case of Met-BLG, most of the DNA was bound when using a charge ratio (+/−) of 1:1, and the band representing the free viral DNA disappeared totally when the charge ratio (+/−) was 2:1. In this case, the entire bound DNA stayed in the wells of the gel without migration. In the case of Et-BLG or Et-ALA, the complete disappearance of the free DNA occurred only when the charge ratio (+/−) was increased to 3:1. However, as it could be better seen on the gels, at a 2:1 charge ratio, the intensity of the band representing the free DNA was higher in the case of Met-ALA than in the case of Et-BLG, showing lower binding capacity for Met-ALA. Such variations in the capacity of esterified milk proteins to form complexes with DNA may

be explained by their different extents of esterification and, hence, their different basicities. The capacity of esterified milk proteins to form complexes with DNA can be classified as follows: 100% Met-BLG (p*I* of 9.5) > 73% Et-BLG (p*I* of 8.5) ≥ 60% Met-ALA (p*I* of 8.0). Generally, this experiment confirms the importance of the concentration of the reacting protein in the viral media. The concentration should be high enough to complex all free DNA; otherwise, the DNA of the virus, which is not complexed with protein, can replicate rapidly.

The viral DNA-binding properties of esterified milk proteins can explain their antiviral mechanism of action. Although bacteriophage M13 is surrounded by a coat protein forming a polymeric protective coat (*44*), it leaves its protein coat during phage disassembly (*45*). Thus, the viral DNA ejected in the bacterial cytoplasm can be a target for the binding of the positively charged esterified proteins. Once the viral DNA is exposed during its usual biological activities, it may be bound by esterified proteins and, hence, subsequent replicating events are affected or totally inhibited depending on the concentration of the esterified protein.

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